

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Investigation of the relationship between voltage-gated ion channels
and ceramides

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I. Preface

The relationship between voltage-gated ion channels and lipids is highly complex. On the one hand, as transmembrane proteins, ion channels are embedded in the membrane. Therefore, large conformational changes associated with the opening of the activation gate during their activation take place within the membrane, in close cooperation with the surrounding lipid molecules. This enables the lipids to influence channel function both through ligand-like, direct molecular interactions and indirectly through affecting membrane biophysical properties that are determined by lipids. It is known that cholesterol, phosphoinositides and polyunsaturated fatty acids exert significant effects on the activation gating of voltage-gated potassium channels (Kv) and the complex effects of these lipids have been proposed to be mediated by both direct interactions and indirect actions through alterations in membrane biophysical parameters as well. However, little is known about the effects of membrane ceramides, including C16-ceramide (Cer), and glucosylceramides, such as C16-glucosylceramide (GlcCer), on the activation gating of voltage-gated ion channels, including Kv1.3, and about the mechanisms of these effects, in spite of the fact that Kv1.3 has been suggested to play key roles, among others, in neurodegenerative diseases in which membrane ceramide levels increase.

The bidirectional nature of the relationship between lipids and ion channels is indicated by the fact that not only can lipids affect the function of voltage-gated ion channels, but the activity of voltage-gated ion channels may also be able to modify lipid levels, including the amounts of ceramide, by influencing their synthesis and degradation. In several cell types, inhibitors of the voltage-gated proton channel (Hv1) have been proposed to reduce cell viability. This has been experimentally demonstrated, among others, in T-lymphocytes, chorion-derived mesenchymal stem cells, myeloid-derived suppressor cells, and various tumor cells. However, this has not been previously investigated in macrophages. Such an effect of Hv1 inhibitors may be important both from the perspective of their therapeutic and side effects as well. Furthermore, it is also known that an increase in cellular ceramide levels is often associated with the induction of cell death. Since many ceramide-synthesizing enzymes exhibit pH-dependent activity, it is possible that changes in intracellular pH caused by Hv1 inhibition may enhance the activity of these enzymes and thereby increase ceramide levels, ultimately leading to cell death. However, the relationship between these two processes has not yet been investigated in detail.

II. Introduction

II./1. Structure, gating and physiological roles of the Kv1.3 voltage-gated potassium channel

Regarding both its structure and function, the Kv1.3 ion channel is one of the prototypes of the superfamily of voltage-gated potassium channels. It is composed of four alpha subunits, each containing six transmembrane helical segments (S1–S6) connected to each other by intra- and extracellular loops. The S1-S4 segments form the voltage-sensor domain (VSD), whereas the S5-S6 segments form the pore domain (PD) of the channel. During activation gating, depolarization of the cell membrane causes the S4 helix of the VSD to slide outward from the plane of the cell membrane toward the extracellular space, owing to its positively charged amino acids. Through the functional coupling between the two domains, this movement is ultimately transmitted to the activation gate, resulting in its opening at the intracellular end of the PD. Physiological roles of Kv1.3 have been described in various cell types. In immune cells, including T- and B-lymphocytes, macrophages, microglia and dendritic cells, the channel plays an important role in cellular activation; in neurons, it contributes to the fine-tuning of the resting and action potentials; and Kv1.3 located in the inner mitochondrial membrane is important in the regulation of apoptosis.

II./2. Inhibitors of the Kv1.3 voltage-gated potassium channel and the therapeutic significance of its inhibition

The two major groups of pharmacological inhibitors of the Kv1.3 ion channel comprise small-molecule and peptide-type inhibitors. Among the small-molecule inhibitors, psoralens represent the most significant group, whose most important members are Psora-4 (5-(4-phenylbutoxy)psoralen) and PAP-1 (5-(4-phenoxybutoxy)psoralen), the most effective psoralen also used in clinical trials. The other major class consists of peptide-type inhibitors. The most significant members of this group include Vm24, also studied by our research group, and dalazatide, already studied in clinical trials. The therapeutic potential of Kv1.3 inhibition has been proposed in autoimmune, neurodegenerative and cancerous diseases. However, to date, none of these inhibitors has entered clinical practice.

II./3. Structure, gating and physiological roles of the Hv1 voltage-gated proton channel

In contrast to the structure of Kv channels, Hv1 is present in the membrane as a homodimer in which each individual subunit is composed of four transmembrane helical segments (S1-S4). The structure of the subunits closely resembles that of the voltage-sensor

domain of K_v channels. However, unlike in K_{vs}, these subunits simultaneously function as voltage sensors and serve as the conduction pathway for the flow of protons directed outward from the cytoplasm under physiological conditions. This is because these channels lack the classical PD described above, formed by the S5-S6 helices. Hv1 is activated when the cell membrane is depolarized, or when the proton concentration in the cytosol increases that is, when the cytosol becomes acidified, or, conversely, when the extracellular space becomes alkalinized. Hv1 has a particularly important role in two cellular functions. The channel is indispensable for the proper functioning of NADPH oxidase and thereby for maintaining the production of reactive oxygen species (ROS) and its other main function is the regulation of intracellular pH.

II./4. General overview of macrophages and the role of Hv1 in macrophages

Macrophages, as essential members of the innate immune system, are highly plastic and heterogeneous cells. Consequently, their differentiation and activation can be triggered by numerous different stimuli. Macrophages differentiate from monocytes entering the tissues. In general, macrophages can be classified into M1, or classically polarized, and M2, or alternatively polarized, subgroups. Activated M1 macrophages produce pro-inflammatory cytokines and mediators, through which they participate in defense against pathogens and tumors, whereas M2 macrophages are rather involved in the resolution of inflammation, the removal of apoptotic cells and debris, and tissue remodeling. Although the above dichotomous classification is widely used in the literature, the M1 and M2 phenotypes should be regarded as the extremes of a continuum of possible functional states, which may dynamically transition into each other depending on microenvironmental factors. The fundamental role of Hv1 has been widely documented in human macrophages, in which the channel has been associated with the regulation of intracellular pH homeostasis and ROS production, and thereby with their antimicrobial and pro-inflammatory activity.

II./5. Inhibitors of the Hv1 voltage-gated proton channel and the therapeutic significance of its inhibition

Various inhibitors of the Hv1 ion channel have been described, which include ions, small-molecule and peptide-type inhibitors. Zn²⁺, the first Hv1 inhibitor described in the literature, can be regarded as a nonspecific inhibitor. Among small-molecule Hv1 inhibitors, guanidinium-based compounds remain one of the most significant groups. Their two most important members are 2-guanidinobenzimidazole (2GBI) and its modified derivative, 5-chloro-2-guanidinobenzimidazole (ClGBI). Owing to its favorable properties, primarily its high

affinity, ClGBI is still the most widely used compound for the pharmacological inhibition of Hv1 and for the experimental investigation of the function and biological significance of the channel. Therefore, we also applied this compound in our experiments. Peptide-type inhibitors include AGAP (Anti-tumor Analgesic Peptide) and Hanatoxin. Numerous pathological conditions are known in which excessive proton channel activity plays a key role and, therefore, its selective inhibition holds considerable therapeutic potential. Accordingly, beneficial effects of Hv1 inhibition have already been proposed in acute lung injury, ischemic stroke, brain and spinal cord injuries, multiple sclerosis and various types of cancer.

II./6. Major membrane biophysical parameters, their measurement and biological significance

Lipids can influence the function of transmembrane proteins, including ion channels, through direct, ligand-like lipid-protein interactions, as well as indirectly, by altering the biophysical properties associated with the molecular order of membranes. The latter include fluidity, hydration and dipole potential, which characterize the structural organization of membranes at different depths and can be studied in living cells using fluorophores sensitive to the local microenvironment and fluorescence-based measurement techniques. The most commonly used methods for this purpose are the determination of TMA-DPH fluorescence anisotropy, Laurdan generalized polarization and di-8-ANEPPS excitation ratio, respectively. These methods make it possible to elucidate the effects of various lipids on membrane organization, thereby also enabling the study of potential indirect effects of lipids on membrane proteins.

II./7. Membrane biophysical characteristics of ceramides

The organization of membranes and thus their biophysical properties are primarily determined by their lipid composition. Due to their characteristic chemical structure and extreme hydrophobicity, ceramides markedly increase the packing density of membrane molecules and the molecular order of bilayers, and they create membrane microdomains with unique physical properties, known as ceramide platforms. Glucosylceramides also generally increase membrane order and contribute to the formation of lipid raft membrane microdomains. Through all these effects, both ceramides and glucosylceramides may participate in the modulation of the functional activity of proteins located in the membrane. However, studies performed in model membranes have suggested that differences may exist in the membrane biophysical effects of ceramides and glucosylceramides, as well as in their magnitude, through

which their effects on proteins may also be distinct. Still, this has not yet been demonstrated in living cells.

II./8. Electrophysiological effects of ceramides on voltage-gated potassium channels

For various members of the Kv superfamily, including the Kv1.3 itself, it has been suggested that ceramides generally exert inhibitory effects on channel function. These effects typically manifest as reduced whole-cell currents, lower single-channel open probabilities and shifts of the conductance–voltage curve toward higher membrane potentials. Nevertheless, it remains essentially completely unexplored through what mechanism of action ceramides exert their effects and which domain of the channels they act on. Moreover, the effects of glucosylceramides on Kv channels are entirely unknown.

II./9. Investigation of the mechanisms of action of lipids

Two-electrode voltage-clamp fluorometry is a unique combination of electrophysiological and fluorescence-based techniques. By measuring ionic currents and simultaneously monitoring fluorescence after labeling a cysteine residue introduced into the VSD of the ion channel by site-directed mutagenesis with a fluorophore, this method enables the simultaneous investigation of the function of the PD and VSD. Therefore, this technique offers an exceptional opportunity to study the complex mechanisms of action of lipids and to determine which domain of the channel is the primary intramolecular target of lipids. In addition, the indirect effects of ceramides and glucosylceramides mediated through changes in membrane biophysical properties can be studied using the above-mentioned environment-sensitive fluorophores. Investigating this is particularly relevant considering the fact that the conformational changes occurring during the activation gating of the channel take place in a region where alterations in lipid abundance may result in fundamental structural rearrangements, as has also been already demonstrated for other ion channels.

II./10. Synthesis and pathophysiological significance of ceramides and their role in cell death

Both ceramides and glucosylceramides can be considered bioactive compounds, meaning that their levels are precisely regulated and changes in their abundance consequently influence the levels and activity of various target molecules. Their physiological amounts are thus essential for normal cellular function. Ceramides are the central compounds in sphingolipid metabolism. Although they can be found only in negligible amounts in the plasma membrane under physiological conditions, they form the basic compounds of more complex sphingolipids.

Their levels may also increase markedly in response to various triggers, particularly stress stimuli, which leads to the activation of signaling processes and, in many cases, triggers pathways leading to cell death. Glucosylceramides are generated from ceramides and serve as precursors of more complex glycosphingolipids and gangliosides. They are also present in large amounts even under physiological conditions, and substantially contribute to the lateral organization of the cell membrane. In accordance with their important biological roles, changes in the levels of these lipids are characteristic of several pathological conditions. The pathophysiologically relevant accumulation of ceramides can be observed in various neurodegenerative disorders, whereas in many tumors, in contrast, ceramide production cannot be induced. Both alterations are typically associated with disturbances in cell death pathways. Elevated glucosylceramide levels also occur in numerous morbidities, including neurodegenerative diseases, lysosomal storage disorders and cancerous diseases, and often contribute to the abnormal function of certain proteins characteristic of these states. Since dysfunction of ion channels is also characteristic of all the aforementioned pathological conditions, elucidating the relationship between these two factors may contribute to a better understanding of these diseases and to the identification of new therapeutic targets as well.

III. Objectives

The research presented in the dissertation was organized around two main questions:

1. *The primary aim of the first study described in the dissertation was to investigate, using modern electrophysiological and membrane biophysical methods, how membrane-embedded C16-ceramide (Cer) and C16-glucosylceramide (GlcCer) modify the activation gating of Kv1.3 ion channel.*

To this end, our objectives were to: i) examine the electrophysiological effects of these lipids on Kv1.3, a prototypical member of the Kv ion channel superfamily; ii) identify the structural element of the channel, that is the VSD or the PD, through which the electrophysiological effects of these lipids are mediated; and iii) support or exclude the contribution of indirect effects mediated by changes in membrane biophysical parameters to the mechanism of action of each lipid. In our two-electrode voltage-clamp fluorometry measurements using the *Xenopus laevis* oocyte expression system and exogenous Cer and GlcCer loading, we determined the main electrophysiological parameters that describe the effects of these ceramides on ionic currents. Examination of changes in the fluorescent signal monitoring VSD movement enabled the identification of the intramolecular target of these lipids, thereby providing insight into their mechanism of action. To determine whether indirect effects arising from changes in lipid levels may contribute to the effects of ceramides, we characterized the membrane biophysical alterations induced by Cer and GlcCer loading in Chinese hamster ovary (CHO) cells. For this purpose, we used fluorescent probes that, in spectrofluorometric or confocal microscopic measurements, are capable of characterizing membrane biophysical changes in different layers of the bilayer. Thus, we characterized alterations in fluidity in the innermost layers, hydration in intermediate regions, and dipole potential at the outermost membrane-water interface.

2. *The primary aim of the second study described in the dissertation was to investigate how inhibition of the Hv1 ion channel affects the viability of human polarized macrophages and to elucidate the role of ceramides in this process.*

To this end, our objectives were to: i) examine, using flow cytometry, how inhibition of the Hv1 ionic current by ClGBI affects viability of polarized macrophages derived from the human THP-1 monocytoid cell line; ii) study how inhibition of the Hv1 ionic current influences

pH-sensitive ceramide synthesis by quantifying ceramide levels in cells polarized in different directions; and iii) demonstrate the direct relationship between the reduction in viability induced by Hv1 channel inhibition and changes in ceramide levels by examining whether blockade of ceramide synthesis, performed simultaneously with channel inhibition, can prevent changes in macrophage viability.

IV. Materials and methods

IV./1. Cells, expression systems and molecular biology used to study the electrophysiological and membrane biophysical effects of ceramides

To study the electrophysiological effects of ceramides, we used the human Kv1.3 (KCNA3, UniProt B2RA23) A309C mutant channel. For mRNA synthesis, the plasmid encoding the mutant channel was linearized with the HindIII enzyme and then transcribed into mRNA using the Invitrogen mMESSAGE mMACHINE T7 Transcription Kit (Thermo Fisher Scientific, Waltham, MA, United States).

For the TEVCF experiments, *Xenopus laevis* African clawed frog oocytes were obtained from EcoCytte Bioscience (Dortmund, Germany). The oocytes were injected with 30–50 nl of ~1 µg/µl mRNA and then incubated at 18 °C for 1-3 days in ND93 solution containing 93 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES and 50 mg/l gentamicin, pH 7.4. All chemicals used for preparing the solutions were purchased from Sigma-Aldrich (St. Louis, MO, United States).

Changes in membrane biophysical parameters induced by ceramide and glucosylceramide loading were examined in the CHO cell line. The cell line was obtained from the American Type Culture Collection (Manassas, VA, United States) and cultured according to its specifications. For spectrofluorometry measurements, cells showing 80-90% confluence were detached by trypsin treatment, whereas for confocal microscopy, the cells were grown in 8-well chambered coverslips (ibidi, Gräfelfing, Germany).

IV./2. Ceramide and glucosylceramide loading of the cell membrane

For ceramide and glucosylceramide loading, C16-ceramide (Cer, d18:1/16:0, N-palmitoyl-D-erythro-sphingosine) and C16-glucosylceramide (GlcCer, d18:1/16:0, D-glucosyl-β-1,1'-N-palmitoyl-D-erythro-sphingosine) were obtained from Avanti Polar Lipids (Alabaster, AL, United States). The lipids were dissolved directly in absolute ethanol that was present at a final concentration of less than 0.5% during treatments. Cer and GlcCer loading was performed at a concentration of 40 µM for 24 hours by diluting the lipids in ND93 solution for electrophysiology or in medium for spectrofluorometry and microscopy. Additional loading concentrations were also used during viability measurements performed in CHO cells. Incubation was carried out at 18 °C for oocytes and at 37 °C for CHO cells.

IV/3. Two-electrode voltage-clamp fluorometry (TEVCF)

For TEVCF measurements, the oocytes were labeled for 30 minutes on ice with 10 μ M 2-((5(6)-tetramethylrhodamine)carboxylamino)ethyl-methanethiosulfonate (TAMRA-MTS, Toronto Research Chemicals, Toronto, ON, Canada). The dye was diluted in a depolarizing solution containing 110 mM KCl, 1.5 mM MgCl₂, 0.8 mM CaCl₂, 0.2 mM EDTA, and 10 mM HEPES, pH 7.1. After staining, following thorough washing with ND93, the oocytes were stored on ice in the dark until the start of the measurement. The pipettes used for the measurements were pulled from GC 150 F-15 borosilicate glass capillaries (Harvard Apparatus, Kent, United Kingdom), and their resistance was 2-3 M Ω . During the measurements, ND93 solution was used as the external solution in the recording chamber, whereas 3 M KCl solution was used as the internal solution in the pipettes.

During TEVCF, the membrane potential of the oocytes was controlled and ionic currents were measured using an Oocyte Clamp OC-725C amplifier (Warner Instruments, Hamden, CT, United States). The intensity of the fluorescent signals was measured using a 40 $\times$, 0.8-NA CFI Plan Fluor Nikon water-immersion objective mounted on a Nikon Eclipse FNI microscope (Nikon, Tokyo, Japan) and a photodiode (PIN-040A; United Detector Technology, OSI Optoelectronics, Hawthorne, CA, United States). To detect the TAMRA-MTS signal, a 545/25 excitation filter, a 565LP dichroic mirror, and a 605/70 emission filter were used. The photodiode signal was amplified with an Axopatch 200A amplifier, and the data, together with the ionic currents, were digitized using an Axon Digidata 1550 interface (Molecular Devices), controlled by the pClamp10 software package (Molecular Devices, San Jose, CA, United States).

IV/4. Analysis of TEVCF data

During TEVCF measurements performed on the Kv1.3 A309C ion channel, ionic currents and fluorescent signals were sampled at a frequency of 5 kHz with filtering at 1 kHz. The magnitude of the fluorescent signals was expressed as the percentage ratio $\Delta F/F$, where ΔF denotes the change in the amplitude of the fluorescent signal and F is the fluorescence baseline measured at the given holding potential. To eliminate distortive effects caused by photobleaching, the average value of the baseline fluorescence intensity showing no voltage-dependent changes was subtracted from the fluorescence data obtained at different membrane potentials. Subsequently, to generate $F_{\text{norm}}-V$ curves, steady-state components of the fluorescent signals were normalized to the maximal intensity, and the resulting values were plotted as a function of the test potential.

To generate current-voltage (I-V) curves, peak currents were determined, corrected for leak current, and the resulting values were plotted as a function of the test potential. Subsequently, the $V_{1/2}$ and k parameters of the conductance-voltage (G-V) curves were determined by fitting the following equation to the I-V curves:

$$I = \frac{V \times G_{max} \times (1 - e^{-\frac{V - E_{rev}}{25}})}{(1 - e^{-\frac{V - E_{rev}}{25}}) \times \frac{1}{1 + e^{-\frac{V - V_{1/2}}{k}}}} \quad (1.)$$

which combines Goldman-Hodgkin-Katz rectification with Boltzmann voltage dependence. In the formula, V denotes voltage, I is current, and the free parameters of the function are maximal conductance (G_{max}), reversal potential (E_{rev}), and the half-activation voltage ($V_{1/2}$) and slope factor (k) of the Boltzmann function. The number 25, derived from the Goldman-Hodgkin-Katz equation, represents the ratio of the product of the universal gas constant and absolute temperature and the product of the ionic charge and the Faraday constant, expressed in mV. Subsequently, for the given test potentials, the normalized G_{norm} values were calculated for each cell using the following function:

$$G(V) = \frac{1}{1 + e^{-\frac{V - V_{1/2}}{k}}} \quad (2.)$$

Here, $G(V)$ denotes the conductance calculated at the given test potential, V is the actual membrane potential, $V_{1/2}$ is the half-activation voltage, and k denotes the slope factor. In measurements performed in oocytes, G is determined differently than in conventional patch-clamp measurements because, due to the size of the oocytes, the intracellular potassium concentration and thus the value of the reversal potential is unknown when a given internal solution is used.

To determine the activation time constants of the ionic currents, a single-component, first-order, saturating exponential function was fitted to the rising phase of the current traces:

$$I = I_0 \times \left(1 - e^{-\frac{t}{\tau_{act}}}\right) + C \quad (3.)$$

where I is the current amplitude, I_0 is the maximal current amplitude, τ_{act} is the activation time constant, and C is a constant, time-independent current component. For the fluorescent signals, the following two-component, saturating exponential function was fitted to the measured data to determine the fast ($\tau_{act,f}$) and slow ($\tau_{act,s}$) time constants:

$$I = I_{0f} \times \left(1 - e^{-\frac{t}{\tau_{act,f}}}\right) + I_{0s} \times \left(1 - e^{-\frac{t}{\tau_{act,s}}}\right) + C \quad (4.)$$

F_{norm} -V curves were quantified by fitting the following Boltzmann function:

$$y = \frac{1}{1 + e^{-\frac{V - V_{1/2}}{k}}} \quad (5.)$$

and the $V_{1/2}$ and k values were determined from fits to individual cells.

To examine the effects of Cer and GlcCer treatments on current amplitudes, peak currents evoked by depolarizing pulses to +40 mV were determined and corrected for leak current. For each cell, these values were then normalized to the daily mean of the leak-corrected currents obtained in control oocytes. Therefore, the resulting relative current amplitudes were independent of possible variability in ionic currents between measurement days, allowing results obtained on different days to be reliably compared.

Electrophysiological data were analyzed using Clampfit (v10; Molecular Devices), SigmaPlot (v10; Systat Software, San Jose, CA, United States), and Excel (Microsoft, Redmond, WA, United States).

IV/5. Investigation of the fluidity and hydration of the cell membrane

To examine membrane fluidity, control CHO cells and those treated with Cer or GlcCer were labeled with 4'-(trimethylammonio)-diphenylhexatriene (TMA-DPH; Sigma-Aldrich). The fluorescence anisotropy (r) of the dye was then determined using a Fluorolog-3 spectrofluorometer (Horiba Jobin Yvon, Edison, NJ, United States) at 37 °C, after excitation at 352 nm and measurement of emission at 430 nm, according to the following formula:

$$r = \frac{I_{vv} - GI_{vh}}{I_{vv} + 2GI_{vh}} \quad (6.)$$

where I_{vv} and I_{vh} are the vertical and horizontal components, respectively, of fluorescence excited by vertically polarized light, and G is an instrument-specific correction factor characterizing the different sensitivity of the detection system to vertically and horizontally polarized light, according to the following formula:

$$G = \frac{I_{hv}}{I_{hh}} \quad (7.)$$

where I_{hv} and I_{hh} are the vertical and horizontal components of fluorescence after excitation with horizontally polarized light.

To examine membrane hydration, cells were labeled with 6-dodecanoyl-N,N-dimethyl-2-naphthylamine (Laurdan; Sigma-Aldrich). The dye was then excited at 350 nm, and its emission detected with a spectrofluorometer at 37 °C at 435 nm (I_{blue}) and 500 nm (I_{red}). Its generalized polarization (GP) was quantified according to the following formula:

$$GP = \frac{I_{\text{blue}} - I_{\text{red}}}{I_{\text{blue}} + I_{\text{red}}} \quad (8.)$$

IV./6. Determination of the dipole potential of the cell membrane

To assess membrane dipole potential, control CHO cells and those treated with Cer or GlcCer were labeled with di-8-ANEPPS (4-(2-[2-[6-(dioctylamino)-2-naphthalenyl]ethenyl)-1-(3-sulfopropyl)pyridinium inner salt; Thermo Fisher Scientific). Images were acquired at the midplane of cells using an LSM880 confocal laser-scanning microscope (Carl Zeiss AG, Jena, Germany). The dye was excited at 458 nm and 514 nm, and the emitted intensity was measured between 584 and 686 nm (I_{blue} and I_{red} , respectively). During quantitative image analysis, the mean value of the fluorescence ratio ($R_{exc} = I_{blue} / I_{red}$) positively correlating with the magnitude of the dipole potential was calculated exclusively from data of membrane pixels identified using a watershed algorithm, after subtraction of background intensities.

IV./7. Investigation of membrane ceramide and glucosylceramide levels

To examine membrane ceramide levels, control CHO cells and those treated with Cer or GlcCer were labeled with 15B4 anti-ceramide antibodies (Sigma-Aldrich), followed by fluorophore-conjugated anti-mouse IgM secondary antibodies (Thermo Fisher Scientific). To determine the amount of glucosylceramide, anti-glucosylceramide primary antibodies (Glycobiotech, Kukels, Germany) and then fluorophore-conjugated anti-rabbit IgG secondary antibodies (Thermo Fisher Scientific) were similarly applied. In both cases, the fluorescence intensity of individual cells was measured using a NovoCyte 3000RYB flow cytometer (ACEA Biosciences, San Diego, CA, United States). During analysis performed in FCS Express (De Novo Software, Los Angeles, CA, United States), the mean fluorescence of cells showing normal morphology, normalized to the control samples, was determined.

IV./8. Investigation of the effects of ceramide and glucosylceramide on cell viability

Control CHO cells and those treated with different concentrations of Cer or GlcCer, already in suspension together with those detached by trypsin detachment, were labeled with Sytox Green Dead Cell Stain (Thermo Fisher Scientific) and fluorophore-conjugated annexin V (Thermo Fisher Scientific) markers to detect necrosis and apoptosis. Subsequently, the fluorescence intensity of individual cells was measured using a NovoCyte 3000RYB flow cytometer, and the fraction of cells negative for both markers and therefore considered viable was quantified relative to the total number of cells in each sample using FCS Express.

IV./9. Culturing and differentiation of THP-1 cells and polarization of macrophages

THP-1 cells (American Type Culture Collection) derived from human acute monocytic leukemia were differentiated into macrophages for 24 hours using 10 ng/ml phorbol-12-

myristate-13-acetate (PMA; Sigma-Aldrich), followed by a 24-hour resting period in PMA absence. Differentiated M0 macrophages were then polarized for 24-48 hours into classical M1 macrophages with 100 ng/ml lipopolysaccharide (LPS; E. coli O111:B4 ultrapure, Sigma-Aldrich) and 20 ng/ml interferon- γ (IFN- γ ; Thermo Fisher Scientific), or into M2 macrophages using 20 ng/ml interleukin-4 (IL-4; Thermo Fisher Scientific) and 20 ng/ml interleukin-13 (IL-13; Thermo Fisher Scientific).

IV/10. Analysis of CD marker expression in polarized macrophages

Differentiated and polarized THP-1 macrophages were detached using Accutase, and their Fc receptors were blocked with human FcR blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany). The cells were then labeled with fluorophore-conjugated anti-CD64, anti-CD71, anti-CD80, anti-CD86 or anti-CD206 antibodies (all from Thermo Fisher). The fluorescence intensity of individual cells was measured using a NovoCyte 3000RYB flow cytometer, and their mean fluorescence intensity was calculated in FCS Express. The absence of nonspecific binding was demonstrated using isotype control antibodies applied under identical conditions.

IV/11. Analysis of effects of ion channel inhibitors on viability of polarized macrophages

Differentiated and polarized THP-1 macrophages were treated for 24 hours with the following ion channel inhibitors: 16-256 μ M 5-chloro-2-guanidinobenzimidazole (ClGBI; Sigma-Aldrich), 20 pM Vm24 scorpion toxin (Alomone Labs, Jerusalem, Israel), 20 nM tetrodotoxin (TTX; Alomone Labs), 1 mM tetraethylammonium (TEA⁺; Sigma-Aldrich), or ZnCl₂ at a nominal concentration of 1 mM (Sigma-Aldrich). To investigate the potential protective effects of pretreatments inhibiting ceramide production against ClGBI-induced toxicity, 1 μ M myriocin, 2 μ M GW4869, or 5 μ M ARC39 (all from Sigma-Aldrich) were added to polarized M0, M1, and M2 macrophages 30 minutes before ClGBI application. The fraction of viable cells was then determined as described in section IV./8, with the difference that adherent cells were detached using Accutase rather than trypsin.

IV/12. Determination of Hv1 expression in polarized macrophages

Differentiated and polarized THP-1 macrophages were detached using Accutase, fixed with methanol (Sigma-Aldrich), and then, after permeabilization in 0.1% Triton X-100 (Sigma-Aldrich), blocked with human FcR blocking reagent and 5% BSA. The cells were then labeled with anti-Hv1 antibodies (Thermo Fisher Scientific), followed by fluorophore-conjugated goat anti-rabbit IgG antibodies in the presence of 1% BSA and 0.1% Triton X-100. The fluorescence

intensity of individual cells was measured using a NovoCyte 3000RYB flow cytometer, and their mean fluorescence intensity was calculated in FCS Express. The absence of nonspecific antibody binding was demonstrated using isotype control antibodies applied under identical conditions.

IV./13. Analysis of time-dependent effects of CIGBI on plasma membrane ceramide levels in polarized macrophages

Differentiated and polarized THP-1 macrophages were treated with 50 or 100 μ M CIGBI for 1, 4, or 18 hours. The cells were detached using Accutase, blocked with human FcR blocking reagent, and then labeled with anti-ceramide antibody followed by fluorophore-conjugated goat anti-mouse IgM antibody. The fluorescence intensity of individual cells was measured using a NovoCyte 3000RYB flow cytometer, and their mean fluorescence intensity was calculated in FCS Express. The absence of nonspecific antibody binding was demonstrated using isotype control antibodies applied under identical conditions.

IV./14. Statistical analysis

All electrophysiological experiments were performed on oocytes obtained from at least four different frogs. During spectrofluorometry, each sample obtained from three independent experiments contained approximately 100,000 cells. In the ceramide-loading experiments, confocal microscopic analysis was performed using images recorded for each treatment from five independent experiments, with an average of 30–40 cells per image. In confocal microscopic measurements aimed at investigating macrophages, the results were obtained from cells derived from five independent experiments. In flow cytometric measurements, each sample obtained from at least three independent experiments contained at least 10,000 cells. During statistical analysis, p values were determined using Tukey's honestly significant difference (HSD) test when preliminary ANOVA analysis revealed significant differences between the groups. Differences were considered significant when $p < 0.05$.

V. Results and discussion

V./1. C16-ceramide and C16-glucosylceramide exert similar electrophysiological effects on the Kv1.3 ion channel, despite targeting different domains within the ion channel, which can be partly explained by the distinct membrane biophysical properties of the two lipids

In our experiments, we first examined the electrophysiological effects of Cer and GlcCer loading by simultaneously recording ionic currents and fluorescent signals representing PD and VSD movements, respectively, which allowed us to determine the steady-state activation and kinetic parameters characterizing Kv1.3 pore opening and VSD activation. We used the TEVCF technique to describe the electrophysiological effects induced by Cer and GlcCer loading and to identify the intramolecular target of the two lipids. For our measurements, the Kv1.3 ion channel carrying the A309C point mutation was expressed in *Xenopus laevis* African clawed frog oocytes. Based on our previous study, this channel has electrophysiological properties similar to those of the wild-type and it is optimally suited for MTS-TAMRA labeling and, consequently, for TEVCF measurements. Our results demonstrated that Cer or GlcCer loading of the cell membrane exerts an inhibitory effect on the activation gating of the Kv1.3 ion channel. Using TEVCF, we showed that although both lipids induce a rightward shift in the G-V curve characterizing the steady-state activation of the channel to more positive membrane potentials, slow the activation kinetics of the current, and reduce current amplitude, these lipids likely exert their effects through different molecular mechanisms. These mechanisms were differentiated with the parallel recording of the fluorescent signal monitoring VSD movement and the ionic currents characterizing the PD. Based on our results, Cer exerts its effect primarily on the VSD, as indicated by the rightward shift of the F-V curves characterizing the steady-state activation of the fluorescent signal in parallel with such changes in the G-V curves, as well as by the slowing of the activation kinetics of the fluorescent signal. In contrast, GlcCer does not induce such alterations in the fluorescent signal, and thus mainly acts directly on the PD. The relevance of the changes observed in response to our treatments is indicated by the fact that the magnitude of the alterations in these parameters was similar to those previously observed for cholesterol by our research group in the same experimental system.

The TEVCF measurements indicate that Cer primarily exerts its effect through the VSD, whereas GlcCer acts directly through the PD. This suggests that the two lipids may influence Kv1.3 gating through different mechanisms of action and distinct intramolecular targets. To determine whether potentially different membrane biophysical effects of Cer and GlcCer contribute to their distinct mechanisms of action, we examined changes in biophysical

parameters characterizing molecular order in the cell membrane after loading living CHO cells with the two lipids. The complex membrane biophysical effects of the two ceramide species were investigated at three different depths of the lipid bilayer by quantifying their effects on fluidity, hydration, and dipole potential, through quantifying TMA-DPH fluorescence anisotropy, Laurdan generalized polarization, and excitation ratio of di-8-ANEPPS, respectively. To examine changes in the plasma membrane, we used short staining periods and rapid measurement times in spectrofluorometry performed with TMA-DPH and Laurdan in order to avoid dye internalization. In contrast, the quantitative image analysis used in di-8-ANEPPS experiments enabled us to examine the fluorescent signals only originating from the plasma membrane and thus to determine the magnitude of the dipole potential exclusively in that region. Our membrane biophysical measurements demonstrated that while both lipids similarly alter membrane organization in the inner layers, as reflected by decreased membrane fluidity and hydration, Cer induces dramatic and unique changes at the membrane-water interface, manifested as a reduced dipole potential in response to Cer loading. This latter, initially unexpected change may paradoxically indicate a highly ordered local microenvironment, which could oppose VSD rearrangement and conformational change during activation gating in this interfacial region.

Studying the effects of ceramides on the activation gating of K_v ion channels, as well as elucidating the potential contribution of indirect membrane biophysical effects, is of remarkable biological significance. Membrane ceramide and glucosylceramide levels are altered in numerous diseases, which may substantially influence ion channel function and even contribute to the pathomechanism of these pathological conditions. For example, elevated ceramide levels and their pathophysiological significance have been widely described in cardiovascular and neurodegenerative disorders, whereas ceramide levels and production are reduced in various tumors, contributing to their resistance to apoptosis. Furthermore, glucosylceramides accumulate pathologically in Gaucher disease and Parkinson's disease, and their elevated levels may also contribute to the development of multidrug resistance in tumors. In addition to their significance in pathological conditions, these lipids play fundamental physiological roles as well. Ceramides are typically present in very low amounts in the cell membrane, but their levels increase markedly in response to certain stress stimuli, such as tumor necrosis factor- α , ionizing radiation and various chemotherapeutic drugs. Ceramide accumulation in turn promotes the initiation of signaling mechanisms leading to various forms of cell death, including autophagy, mitophagy, apoptosis, and cellular senescence. In contrast, glucosylceramides are generally relatively abundant in the cell membrane and are major

components in the lateral organization of lipid bilayers. Considering that Kv channels are important players in practically all of the pathological conditions and physiological cellular processes described above, elucidating the relationship between altered lipid levels and changes in conductive functions of Kv channels may substantially contribute to a better understanding of these disorders, while also enabling identification of new molecular targets and development of therapeutic approaches.

V./2. Inhibition of the Hv1 proton channel reduces the viability of human THP-1-derived polarized macrophages, and the accumulation of ceramides produced by pH-sensitive acid sphingomyelinase is a key step in the process

In the second part of the dissertation, we investigated the effect of Hv1 ion channel inhibition on the viability of polarized human macrophages. In our experiments, we demonstrated that ClGBI, still the most widely used Hv1 inhibitor, reduces the viability of polarized macrophages derived from the human THP-1 cell line in a dose-dependent manner when applied at biologically relevant concentrations. Based on our results, macrophages polarized in different pathways differ in their sensitivity: M1 cells proved to be the most sensitive, whereas M2 macrophages were the least sensitive to the compound. Our findings suggest that ClGBI indeed elicits this effect primarily through Hv1 inhibition because: i) the ClGBI sensitivity of M0, M1 and M2 macrophages shows a strong positive correlation with the level of Hv1 expression and M1 macrophages, which express the channel at the highest level, are the most sensitive to the compound; ii) inhibitors of other relevant ion channels expressed in macrophages and potentially also affected by ClGBI do not cause any substantial reduction in viability; and iii) Zn^{2+} , another nonspecific Hv1 inhibitor, reduces the viability of differently polarized macrophages in a manner similar to ClGBI.

In our further experiments, we showed the substantial role of ceramides in the reduction in viability induced by Hv1 inhibition. This was supported by the observation that the amount of ceramide in the membrane increased in a time-dependent manner in response to ClGBI. Interestingly, even in the absence of ClGBI treatment, higher ceramide levels were found in M1 macrophages compared with M0 and M2 cells. This may result from the overexpression and activation of neutral and, particularly, acid sphingomyelinase, which are also typical features of classical M1 polarization. The intrinsically higher ceramide level may also increase the propensity of M1 macrophages for ceramide overproduction and thus contribute to the greater sensitivity of these cells to the viability-reducing effects of Hv1 inhibitors. Furthermore, to clearly demonstrate the causal role of increased ceramide levels in this process, we examined

the potential contribution of ceramide-producing enzymes using their specific inhibitors. For this purpose, serine palmitoyltransferase was inhibited with myriocin, neutral sphingomyelinase with GW4869, and acid sphingomyelinase with ARC39. Among the inhibitors tested, ARC39 provided substantial protection against CIGBI-induced cell death since it reduced both Hv1 inhibition-elicited toxicity and ceramide accumulation in the plasma membrane by 50–70%, particularly in M1 cells. Overall, our results suggest that Hv1 inhibition leads to a polarization-dependent reduction in viability of polarized macrophages. Ceramides that accumulate in the membrane as a result of the activity of pH-sensitive acid sphingomyelinase and subsequently induce cell death may play a key role in this process.

Considering that Hv1 inhibition may have therapeutic potential in various human disorders, including neuroinflammatory and tumor diseases, elucidating its effects on the viability of macrophages polarized through different pathways is of crucial importance with respect to both the therapeutic and the possible side effects of channel inhibitors. The use of Hv1 blockers appears highly promising in pathological conditions associated with neuroinflammatory processes. In microglia, the Hv1-mediated proton efflux is essential for counteracting the acidosis arising due to the charge-transferring activity of the ROS-producing NADPH oxidase. Therefore, the Hv1-mediated proton efflux enables the maintenance of elevated ROS production, which plays a decisive role in neuroinflammatory processes and in the consequent neuronal death as well. Accordingly, in mouse disease models, the functional absence of Hv1 reduced neuronal death, brain damage and motor deficits in ischemic stroke, white matter injury induced by cerebral artery stenosis, demyelination in models of multiple sclerosis, as well as neuronal apoptosis and pain hypersensitivity in models of traumatic brain and spinal cord injury. Considering the functional similarities between microglial cells and macrophages, the molecular mechanism of Hv1 inhibition-induced reduction in viability elucidated in our experiments may also be relevant to the beneficial effects described above. In the future, however, the possible adverse effects of Hv1 blockers on macrophage functions must also be taken into account when applied in the above indications. Malignant tumors represent another group of diseases in which Hv1 inhibitors may potentially be used beneficially. Rapidly proliferating neoplastic cells are generally mildly depolarized and acidotic due to the characteristic metabolic shift often referred to as the Warburg effect. Moreover, these cells typically express elevated Hv1 levels, which is highly advantageous, as the channel provides an efficient mechanism for removing protons from the cytoplasm. This mechanism thus protects against intracellular acidosis and, at the same time, creates an acidic extracellular microenvironment that favors degradation of the extracellular matrix facilitating invasion, and

also contributes to the evasion of immune activation. Hv1 overexpression is consistently associated with disease severity and poor prognosis in various tumors, including breast cancer and colorectal carcinoma. Consistently, pharmacological inhibition of the channel reduced the viability and migration of tumor cells in *in vitro* experiments. At the same time, according to our results, Hv1 inhibition may also reduce the viability of polarized macrophages, primarily in M1 cells that typically possess antitumor activity. This may thereby favor the dominant intratumoral presence of M2-like tumor-associated macrophages, aggravating the disruption of the M1-M2 balance and contributing to the protection of tumor cells against the immune system. These potential effects should be studied in the future, as such investigations may indicate that the application of Hv1 inhibitors requires a personalized, selective approach specifically targeting tumor cells.

VI. New findings

1. Using two-electrode voltage-clamp fluorometry (TEVCF), we demonstrated that loading cells with either exogenous C16-ceramide (Cer) or C16-glucosylceramide (GlcCer) induces a rightward shift in the conductance-voltage (G-V) curves characterizing the steady-state activation of Kv1.3 ion channels expressed in *Xenopus laevis* oocytes, and both lipids slow the activation kinetics of the ionic current and decrease its amplitude. Therefore, the two lipids exert similar, overall inhibitory effects on the activation gating of the channel.
2. Based on our results, Cer exerts its effects through acting on the voltage-sensor domain (VSD), as indicated by the parallel rightward shift of the fluorescence intensity-voltage (F-V) curves that characterize the steady-state activation of the fluorescent signal measured during TEVCF experiments, together with the G-V curves, as well as by the slowing of the activation kinetics of the fluorescent signal.
3. In contrast, GlcCer does not alter these parameters of the fluorescent signal and therefore, it primarily exerts its effect directly through the pore domain (PD), without modifying the movement of the VSD.
4. When investigating the underlying mechanism of action of the two lipids, using probes sensitive to local microenvironmental parameters and fluorescence-based techniques in Chinese hamster ovary cells, we showed that although both lipids increase membrane order in the deeper membrane layers in a similar manner and to a similar extent, Cer is capable of inducing a robust structural rearrangement at the membrane-water interface, which may inhibit VSD movement during channel activation.
5. We demonstrated that 5-chloro-2-guanidinobenzimidazole (ClGBI), an inhibitor of the Hv1 ion channel, reduces the viability of macrophages derived from the human THP-1 cell line in a dose- and polarization-dependent manner. Based on our flow cytometry results, classically polarized M1 cells proved to be the most sensitive, whereas M2 macrophages were the least sensitive to the compound.
6. We found that ClGBI exerts this effect through inhibiting Hv1, since the extent of reduction in cell viability correlates well with the level of Hv1 expression in the cells. Furthermore, selective inhibitors of other ion channels present in macrophages do not cause a substantial decrease in viability.
7. We also showed that membrane ceramide accumulation in the cells plays a key role in the viability-reducing effect of ClGBI, because the compound not only increases

ceramide levels, but its cytotoxic effect can be significantly attenuated by a simultaneous inhibition of acid sphingomyelinase, an enzyme responsible for ceramide synthesis, as well.

VII. Summary

The relationship between voltage-gated ion channels and lipids is bidirectional. On the one hand, ion channels are transmembrane proteins, therefore, major structural rearrangements resulting in activation are subject to modulatory actions by the lipids of the cell membrane. On the other hand, ion channels can influence numerous cellular processes, thereby indirectly fine-tuning cellular lipid levels. Although ceramides play very important physiological and pathophysiological roles, their potential interactions with voltage-gated ion channels have not been previously studied in detail.

In the first part of the dissertation, we investigated how C16-ceramide (Cer) and C16-glycosylceramide (GlcCer) influence the activation gating of the Kv1.3 ion channel. Using two-electrode voltage-clamp fluorimetry, we found that following exogenous loading of the cells, both lipids exert similar, inhibitory effects on parameters characterizing the channel's pore domain (PD), while only Cer has a similar effect on voltage-sensor domain (VSD) movement. These suggest that the two lipids have distinct intramolecular targets within the ion channel, namely Cer primarily targets the VSD, while GlcCer the PD. Using environment-sensitive fluorophores and fluorescence-based methods, we demonstrated that while Cer and GlcCer similarly increase molecular order in the inner hydrophobic regions of the lipid bilayer, Cer uniquely induces a strong molecular rearrangement at the membrane-water interface, where, during depolarization, the S4 segment of the VSD, containing positively charged amino acids, slides outward from the plane of the membrane. This distinct membrane biophysical effect may explain the unique mechanism of action of Cer, which differs from that of GlcCer, by primarily targeting the VSD.

In the second part of the dissertation, we demonstrated using flow cytometry that the most widely used Hv1 proton channel inhibitor, 5-chloro-2-guanidinobenzimidazole (ClGBI), reduces the viability of polarized macrophages derived from human THP-1 cells at biologically relevant doses, with M1 macrophages being the most sensitive and M2 cells the least sensitive to effects of the compound. The specific effects of ClGBI mediated by Hv1 inhibition are indicated by the fact that the sensitivity of polarized macrophages correlates well with the Hv1 expression levels of the cells. Furthermore, among other ion channel inhibitors relevant to macrophages, only Zn^{2+} , another Hv1 inhibitor, exhibited similar effects. Our results also highlight that inhibition of the channel results in excessive ceramide production via the action of the pH-sensitive acidic sphingomyelinase, which eventually culminates in cell death.

VIII. Publications



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Registry number: DEENK/234/2026.PL
Subject: PhD Publication List

Candidate: Bence Cs. Szabó
Doctoral School: Doctoral School of Molecular Medicine
MTMT ID: 10098535

List of publications related to the dissertation

1. Kovács, T., **Cs. Szabó, B.**, Kothalawala, R. C., Székelyhidi, V., Nagy, P., Varga, Z., Panyi, G., Zákány, F.: Inhibition of the HV1 voltage-gated proton channel compromises the viability of human polarized macrophages in a polarization- and ceramide-dependent manner. *Front. Immunol.* 15, 1-18, 2024.
DOI: <http://dx.doi.org/10.3389/fimmu.2024.1487578>
IF: 5.9
2. **Cs. Szabó, B.**, Szabó, M., Nagy, P., Varga, Z., Panyi, G., Kovács, T., Zákány, F.: Novel insights into the modulation of the voltage-gated potassium channel KV1.3 activation gating by membrane ceramides. *J. Lipid Res.* 65 (8), 1-17, 2024.
DOI: <http://dx.doi.org/10.1016/j.jlr.2024.100596>
IF: 4.1





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List of other publications

3. Szabó, M., **Cs. Szabó, B.**, Kurtán, K., Varga, Z., Panyi, G., Nagy, P., Zákány, F., Kovács, T.: Look Beyond Plasma Membrane Biophysics: revealing Considerable Variability of the Dipole Potential Between Plasma and Organelle Membranes of Living Cells.
Int. J. Mol. Sci. 26 (3), 1-20, 2025.
DOI: <http://dx.doi.org/10.3390/ijms26030889>
IF: 4.9 (2024)

Total IF of journals (all publications): 14,9

Total IF of journals (publications related to the dissertation): 10

The Candidate's publication data submitted to the Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

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